High molecular weight hyaluronan and stroma-embedded factors of radiation--sterilized amniotic membrane stimulate proliferation of HaCaT cell line *in vitro*

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Abstract

The amniotic membrane may be considered as an attractive dressing material. However, the application of a fresh amnion in clinical practice meets several obstacles, mainly its limited availability, and possible risk of infectious factors transmission. Therefore, different procedures of sterilization and preservation of the amniotic membrane have been developed. Although these procedures destroy all viable components of tissue, the biostatic amniotic dressing may still display some beneficial properties. The aim of the study was to explain a possible mechanism of its action using in vitro HaCaT cell proliferation assay and biochemical analysis of accelerated electron beam-sterilized amniotic membrane dressing. The presence of biologically active factors in the amnion samples was assessed using protein microarray, whereas the amount and quality of hyaluronan was verified using polyacrylamide gel electrophoresis/silver staining method.

The sterilized amniotic membrane stimulated HaCaT cells proliferation in vitro. Interestingly, although hyaluronidase itself did not reveal toxicity against HaCaT cells, this effect was partially reduced by addition of hyaluronidase to the culture. As expected, a gel electrophoresis has confirmed the presence of high molecular weight hyaluronan polymers in amniotic dressing. Moreover, the sterilization procedure did not significantly influence the amount and quality of these molecules.

A protein microarray analysis of radiation-sterilized amniotic membranes revealed significant amounts of various proliferation-stimulating factors, including epidermal growth factor (EGF), angiogenin (ANG), insulin-like growth factor-binding protein (IGFBP) and serpin E1. It is plausible that all of them, together with high molecular weight hyaluronan polymers, could contribute to the proliferation-stimulating action of irradiated amniotic membrane dressing.

Key words: *amniotic membrane, HaCaT cell line, hyaluronan, protein microarray, cytokines.*

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Introduction

The amnion is one of the fetal membranes that surrounds and protects the embryo. The amniotic epithelial cells produce numerous growth and differentiation cytokines that may promote cell proliferation and differentiation. It has been proven, that big amounts of the hyaluronic acid (HA) in the stromal layer of amnion may facilitate cell migration and tissue regeneration [1]. Moreover, high molecular weight hyaluronan (HMWH) displays some anti-inflammatory/immunosuppressive properties [2]. Therefore, the amniotic membrane might be considered as an attractive dressing material. The range of clinical applications for the amniotic membrane still expands and

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includes the reconstructive ocular surgery [3], treatment of severe skin burn [4], or a substitution of pleura [5] or pericardium [6]. Some attempts were also made to use the amniotic membrane dressing in a treatment of hard-to-treat venous leg ulcers.

Although the amniotic membrane is, due to its composition, an ideal surface for migration, adhesion, growth and differentiation of epithelial cells, nevertheless the application of fresh amnion in a clinical practice meets several obstacles. The first, the amniotic membrane itself is a very fragile tissue, prone to mechanical damage, therefore it requires some supporting layer, e.g. nylon net, for the application. The second, the availability of fresh amnion specimens may be limited at the time of intended application, since they must be collected directly after the delivery. Furthermore, a possible risk of transmission of viral or bacterial infections obliges to a microbiological and serological screening of donor woman, and may require effective sterilization procedure of presumable dressing material.

Due to issue mentioned above, various procedures of the preparation, sterilization and long-term storage of amniotic membrane samples have been developed. Obviously, the sterilization procedure eliminates all viable components of the amnion dressing, including cytokine-producing cells. Furthermore, possibly, it may damage various macromolecules, including components of the extracellular matrix, thus affecting biological properties of the tissue. Interestingly, our recent studies have shown that application of the deep frozen, accelerated electron beam-irradiated, amniotic membrane dressing in patients with hard-to-treat venous leg ulcers resulted in significant acceleration of re-epithelialization and shortening of wound healing time. However, the molecular background of observed beneficial effect of the amniotic membrane remains unclear. Therefore, the aim of the current study was to explain this mechanism using in vitro tests and biochemical analysis of radiation-sterilized amnion dressing samples.

Material and methods

Amniotic membrane preparation

The amnion samples were collected, prepared and sterilized using accelerated electron beam (35 kGy), as described by Tyszkiewicz *et al.* [7]. The freshly prepared, non-sterilized amnion specimens were used as a control.

For the hyaluronan electrophoresis, samples (approximately 1 cm^2 each) were prepared by their digestion with pronase from Streptomyces griseus (12 U/ml in 0.4 M NaCl) at 37°C, overnight. Then, half of each sample was additionally digested with bovine testicular hyaluronidase (200 U/ml) at 37°C, for 20 minutes, whereas the remaining volume was kept on ice, until being used for electrophoresis.

For an analysis of biologically active proteins the amniotic membrane samples (approximately 1 cm^2 each) were homogenized in 1 ml of ice-cold 1% Triton X-100 in PBS, with a broad range protease inhibitors cocktail (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany) in a glass homogenizer. The samples were centrifuged at 13 500 rpm for 5 min, and supernatants were transferred into fresh tubes for immediate use.

The MTT test

The HaCaT keratinocyte cells were seeded onto the 24 flat-well plates, 4×10^4 cells per well, and shortly incubated to allow their attachment to a plate surface. Then, amniotic membrane specimens, approx. 1 cm^2 each, were inserted into respective wells and cultured together with cells in standard conditions. The cultures without amnion specimens were used as a control. Subsequently, HaCaT cells were cultured with the combination of amniotic membrane or serial dilutions of purified hyaluronan (0.1-10 µg/ml), with, or without addition of hyaluronidase, at the concentration of 20 U/ml. The cell proliferation was assessed after 24 h, using standard MTT assay, as described elsewhere [8].

Hyaluronan electrophoresis

The semi-quantitative analysis of hyaluronan amount and molecular size in pronase-digested amniotic membrane homogenates was performed according to modified procedure described by Ikegami-Kawai and Takahashi [9]. Briefly, samples were run in 15% acrylamide/bis-acrylamide gel, at a constant voltage of 250 V for 25 min in 0.1 M Tris/borate/EDTA buffer in a cold room. The 200 ng of HA, digested with bovine testicular hyaluronidase (200 U/ml), was used as a reference sample. The gels were then stained with 0.005% alcian blue in 2% ethanol and 1% acetic acid for 30 min, in the dark, followed by their destaining in a water, for a next half an hour. Afterwards, the gels were subjected to silver staining, using the silver stain kit (Bio-Rad, Hercules, CA), according to the manufacturer's protocol.

Protein microarray

The assay was performed using the Proteome Profiler kit by R&D Systems, as described in manufacturer's protocol. The 200 µl of standardized amniotic membrane homogenates were mixed with a cocktail of biotinylated detection antibodies and applied onto nitrocellulose membranes spotted with respective capture antibodies. After the overnight incubation, at 8°C, the membranes were washed and then incubated with streptavidin-horseradish peroxidase conjugate, followed by use of the chemiluminescence detection system (ImmunoCruz Luminol Reagent, SantaCruz Biotechnology Inc., Santa Cruz, CA). Finally, the membranes were exposed to the X-ray film (Agfa-Geavert, Mortsel, Belgium) for 15-30 min, to achieve an optimal intensity of the signal. After development the film was scanned, and the optical density of each

analyzed spot was assessed using GelWorks 2D software (UVP, Cambridge, UK).

Results

The HaCaT keratinocyte cell line, when cultured in a presence of either fresh, or radiation-sterilized amniotic membrane, displayed an increased proliferation rate, as compared to control HaCaT cells in medium alone (not shown). However, due to the significant mitochondrial activity of epithelial cells in a fresh amnion, MTT assay did not allow a proper evaluation of HaCaT proliferation. Therefore the further assessment was performed only with sterilized, biostatic amnion samples (Fig. 1).

Interestingly, the observed proliferation-stimulating effect of amniotic membrane was attenuated by the addition of hyaluronidase to the culture, although hyaluronidase itself did not reveal any toxic influence on HaCaT cells (Fig. 1). Similarly, HaCaT cells, when cultured in a presence of purified hyaluronic acid in a concentration range 0.1-10 µg/ml, displayed an increased proliferation rate (not shown). However, the presence in a culture of both, hyaluronan (5 µg/ml) and hyaluronidase (20 U/ml), resulted in significant decrease of HaCaT cells proliferation, even more pronounced, than in case of hyaluronidase-treated amnion-containing samples (Fig. 1).

The molecular weight estimation of hyaluronan polymers and a semiquantitative analysis of its amount in amnion specimens were done by a comparison between radiation-sterilized and fresh amnion samples. Polyacrylamide gel electrophoresis followed by alcian blue/silver staining method has revealed that radiation-sterilized amniotic membrane samples contained significant amounts of HA molecules. When compared to a fresh amnion, the deep frozen, irradiated amniotic samples did not differ significantly regarding amounts of hyaluronan polymers, whereas their approximate molecular size was slightly decreased (Fig. 2). The hyaluronidase treatment of amnion samples resulted in extensive fragmentation of their HA and generation of ladder-like pattern of smaller oligomers, similar to that, observed in hyaluronidase-treated purified hyaluronan (Fig. 2).

The protein microarray assessment of amnion specimens has revealed significant amounts of several cytokines and growth factors, engaged in tissue regeneration and wound healing. There were detected: epidermal growth factor (EGF), interleukin 8 (IL-8), angiogenin (ANG), insulin-like growth factor-binding proteins (IGFBP) 1, 2 and 3, CD26 and serpin E1. Noteworthy, it has been found, that the amount of mentioned factors visibly differed between amnion samples prepared from various donors (Fig. 3). Nevertheless, the accelerated electron beam-irradiation procedure did not influence significantly the amounts of respective proteins in corresponding samples (Fig. 4).

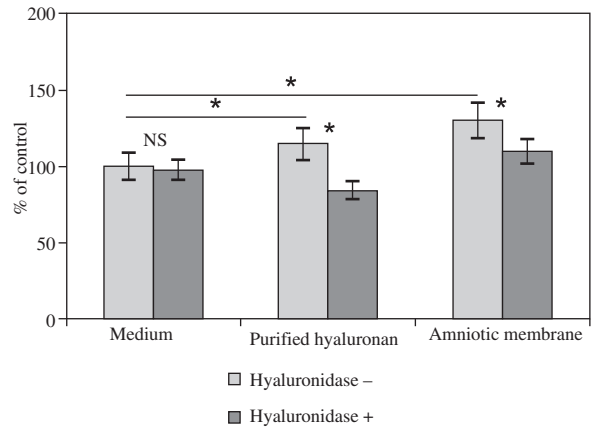

Fig. 1. The proliferation of HaCaT cells, measured in MTT assay after 24 hours incubation in the presence of purified hyaluronan, or amnion sample, and with, or without addition of hyaluronidase. The cell proliferation was expressed as the percentage of MTT-reducing activity of control group, maintained in culture medium alone. The bars represent mean values ± SD, from three independent experiments; asterisks mark the differenced considered as statistically significant ($p < 0.05$); NS – non significant; by Wilcoxon matched pair test

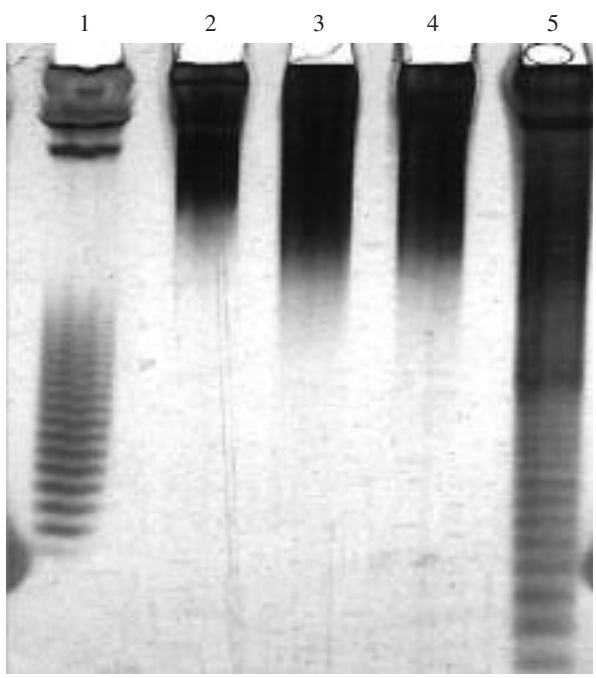

Fig. 2. The representative result of hyaluronan polyacrylamide gel electrophoresis followed by alcian blue/silver staining: 1) purified, hyaluronidase-digested hyaluronan; 2) fresh amnion; 3) frozen amnion; 4) radiation-sterilized, frozen amnion; 5) sterilized amnion, treated with hyaluronidase

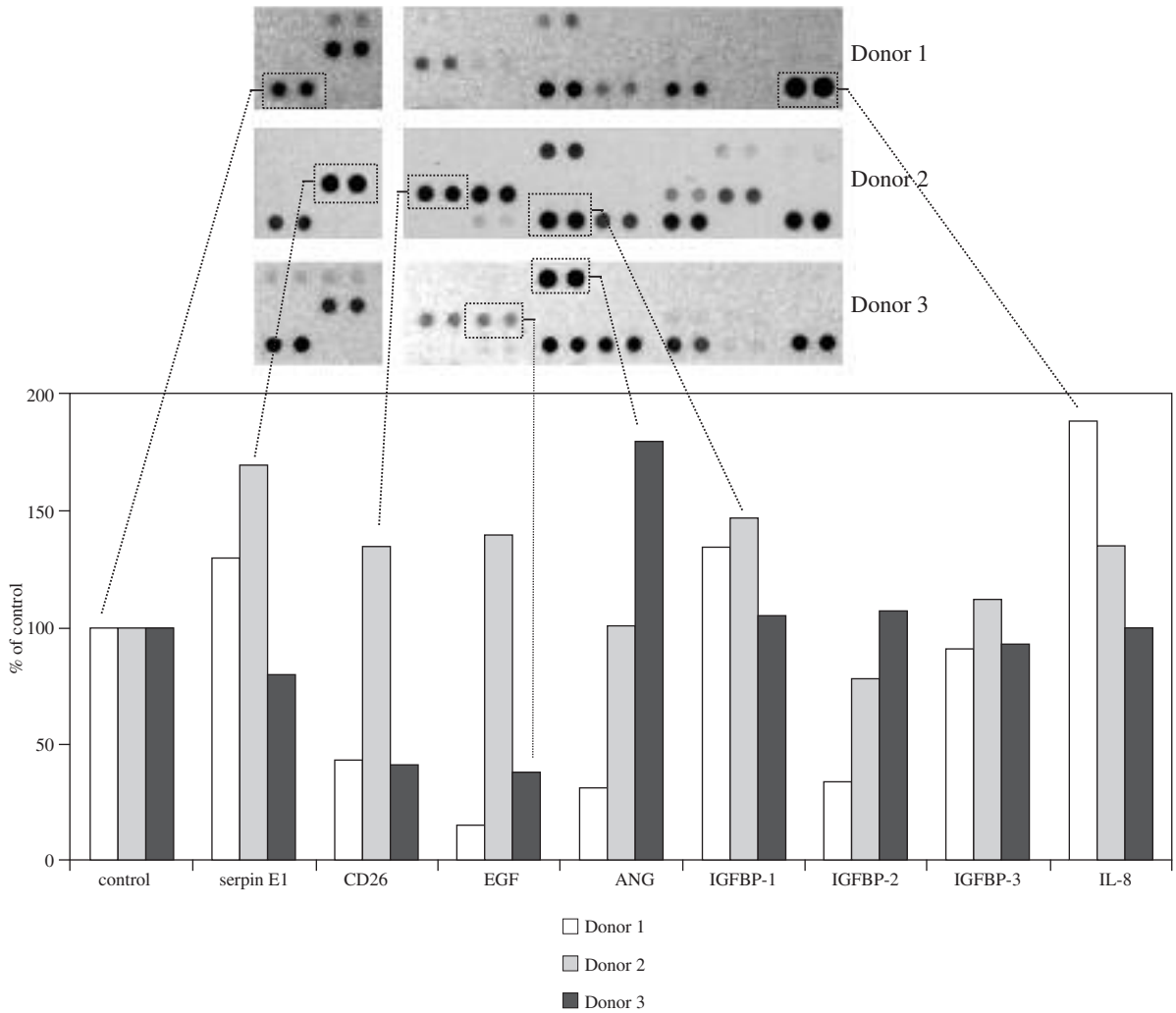

Fig. 3. The protein microarray analysis of radiation-sterilized amnion samples from three various donors. The black/grey dots (in duplicates) represent biologically active factors found in amnion samples. The signal intensity corresponds to the amount of respective proteins, that was adjusted to the signal of positive control and shown on the graph

Discussion

The successful wound healing requires strictly controlled amount and turnover of various extracellular matrix (ECM) components. Among them the significant role in wound healing plays hyaluronic acid [2]. It is a main glycosaminoglycan of the basement membrane, synovial fluid, vitreous of an eye, cartilage and skin. The hyaluronan exists in its native form as a very long polymer, with a huge molecular weight reaching 4 MDa. This polymer, known as the high molecular weight hyaluronan (HMWHA), may be damaged by various types of enzymes, especially hyaluronidases, but also reactive forms of oxygen or mechanical forces to form low-molecular-weight particles of HA (LMWHA). It has been found that LMWHA molecules tend to accumulate at the site of tissue injury, e.g. in area of inflammatory response [10]. Interestingly, recent studies have shown that HA is not only a structural element of the ECM, but it also displays some regulatory properties, that can considerably influence a cell behavior [11]. It has been proven that HMWHA and LMWHA may display an opposite influence on the cells involved in tissue remodeling. The native polymeric hyaluronan reveals anti-inflammatory and immunosuppressive properties. It has been postulated that large polymers of HA may act as a mechanical barrier for the mediators of inflammation thus preventing respective receptors activation [12, 13]. On the other hand, it has been shown that HMWHA downregulated pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) production, possibly by inhibiting the activity of NF-κB, in a T-cell mediated tissue injury model [14, 15].

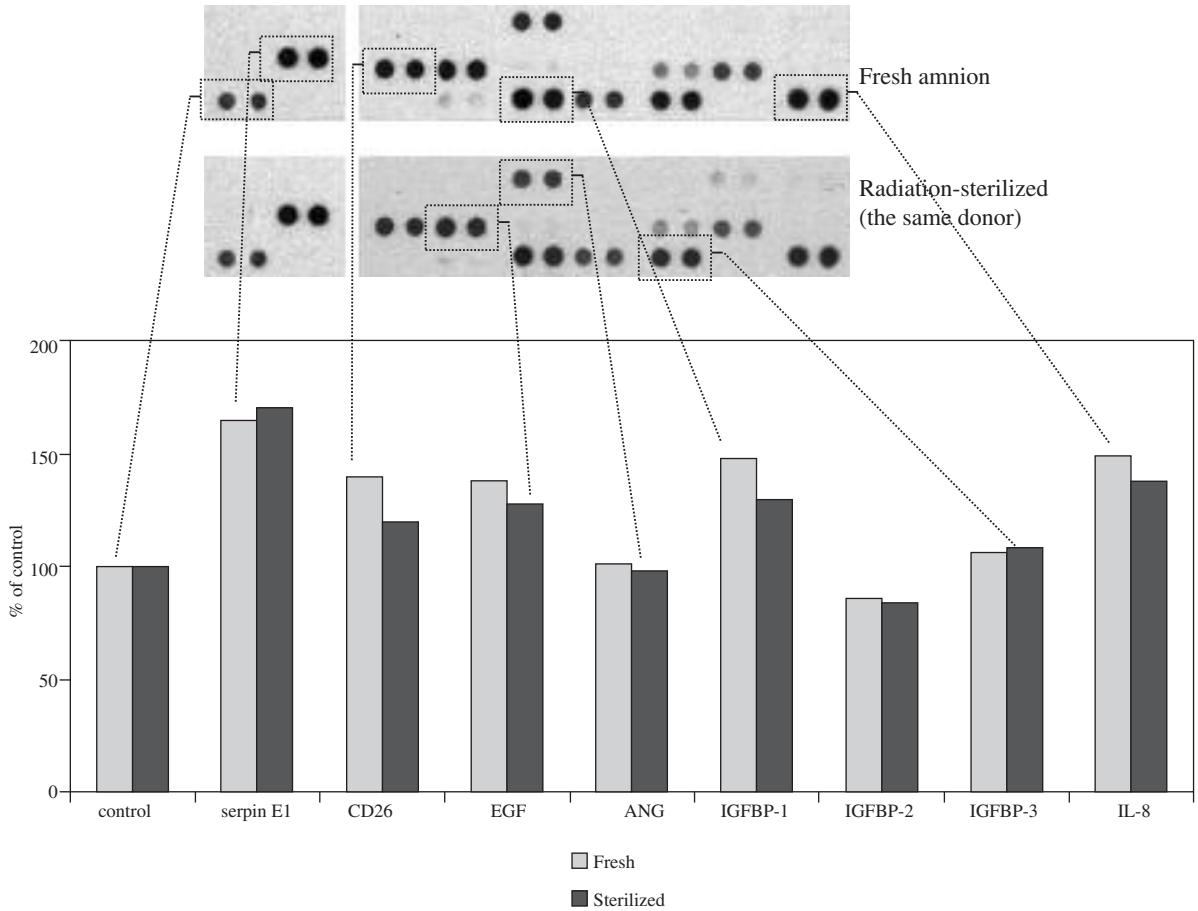

Fig. 4. The example of comparison between fresh and radiation-sterilized amnion samples from the same donor. The results of protein microarray analysis are shown on the graph

In keratinocytes HMWHA prevented the apoptosis induction, whereas in macrophages and neutrophils it inhibited their phagocytic activity [16]. Unlike the large polymer, LMWHA promoted the inflammatory response by binding to the Toll-like receptors (TLR), on various cell types. Toll-like receptors activation resulted in production of numerous pro-inflammatory cytokines and chemokines, thus leading to a state of chronic inflammation [17].

The results of polyacrylamide gel electrophoresis of pronase-digested amnion samples have shown that accelerated electrons-sterilized amniotic dressings still contain significant amounts of HMWHA, that could effectively support a wound healing. Furthermore, the biological importance of this component was confirmed by experiments with hyaluronidase-treated amnion samples, as well as with HaCaT cells *in vitro* cultured with purified HA.

Interestingly, although the hyaluronidase treatment reversed the proliferation-promoting effect of both – purified hyaluronan and native HA in amniotic membrane, the last one still displayed some remaining stimulatory activity. This observation suggested that, in addition to HMWHA, some other factor(s) could presumably contribute to this effect.

It has been proven that amniotic epithelial cells produce numerous biologically active factors, involved in tissue regeneration and wound healing. At least some of them may remain bound to the ECM components in the stromal layer. To verify this hypothesis, the assessment of selected cytokines and factors with cell proliferation-stimulating potential has been performed using protein microarray. It has been found that amnion samples, both, fresh and radiation-sterilized, contained considerable quantities of several wound healing-promoting factors, among them: EGF, IL-8, angiogenin, IGFBPs, CD26, and serpin E1.

Previous studies have demonstrated that EGF and IL-8 promoted reepithelialization by increasing keratinocytes migration and proliferation rate [18, 19]. Clinical trials have shown that topical EGF application signifi-

cantly increased re-epithelialization and shortened healing time in patients with venous and diabetic foot ulcers [20].

Angiogenin, a plasma protein belonging to the ribonuclease A (RNase A) superfamily, is considered to be one of the most potent inducers of angiogenesis *in vivo*. Besides its beneficial role in wound healing, ANG is also implicated in chronic heart failure, asthma and tumor angiogenesis [21, 22]. Interestingly, it was suggested that ANG may reveal some antimicrobial properties, however, these data seem to be not consistent and require further verification [23].

Insulin-like growth factor-binding proteins are family of widely distributed proteins, which bind insulin-like growth factors (IGFs) with a high affinity. They regulate half-life, availability and activity of IGFs. It has been proven, that wound healing was more effective, when a topical administration of IGF-1 was combined with either IGFBP-1, or -3. Interestingly, IGFBPs are also thought to display some IGF-independent activities. They include regulation of cell migration and controlling of keratinocytes apoptosis, presumably through the IGFBPs interaction with integrins, or TGF-β receptors [24].

Both proteinases: serpin E1, also known as a type-1 plasminogen activator inhibitor (PAI-1), and CD26/dipeptidil-peptidase (DPP) IV, are also able to facilitate migration of in vitro cultured cells [25, 26]. In *in vivo* studies mRNA and protein levels of serpin E1 increased rapidly in experimentally created wounds and remained elevated until the keratinocytes formed a monolayer on the damaged skin surface [27]. It was demonstrated that sustained high level of serpin E1 was essential to support the long-term cell motility in a wound bed, possibly by inhibition of plasmin generation [28].

It is plausible, that all of the mentioned above biologically active factors might contribute to the stimulatory effect of the amnion on HaCaT proliferation, observed in our MTT assay, even after hyaluronan digestion. It is noteworthy that, in contrast to other study [29], we did not observe any significant impact of our preparation method on the content of selected pro-angiogenic factors in amnion specimens. Taken together, results of present study may explain in some extent our previous observation, that accelerated electron beam-sterilized amnion dressing, although does not contain any viable components, still possesses strong biological activity and may be used as valuable dressing in various clinical applications (in preparation).

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